LACTB is a tumour suppressor that modulates lipid metabolism and cell state

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Abstract: Post-mitotic, differentiated cells exhibit a variety of characteristics that contrast with those of actively growing neoplastic cells, such as the expression of cell-cycle inhibitors and differentiation factors. We hypothesized that the gene expression profiles of these differentiated cells could reveal the identities of genes that may function as tumour suppressors. Here we show, using in vitro and in vivo studies in mice and humans, that the mitochondrial protein LACTB potently inhibits the proliferation of breast cancer cells. Its mechanism of action involves alteration of mitochondrial lipid metabolism and differentiation of breast cancer cells. This is achieved, at least in part, through reduction of the levels of mitochondrial phosphatidylserine decarboxylase, which is involved in the synthesis of mitochondrial phosphatidylethanolamine. These observations uncover a novel mitochondrial tumour suppressor and demonstrate a connection between mitochondrial lipid metabolism and the differentiation program of breast cancer cells, thereby revealing a previously undescribed mechanism of tumour suppression.

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LACTB, a tumor suppressor that modulates lipid metabolism and differentiation

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SUMMARY:

Post-mitotic, differentiated cells exhibit a variety of characteristics that contrast with those of actively growing neoplastic cells, such as expression of cell-cycle inhibitors and differentiation factors. We hypothesized that the gene expression profiles of such cells would reveal the identities of genes that function as tumor suppressors. Here we show using *in vitro* and *in vivo* studies that the mitochondrial protein LACTB is a potent inhibitor of the proliferation of breast cancer cells. Its mechanism of action involves alteration of mitochondrial lipid metabolism and differentiation of breast cancer cells. This is achieved, at least in part, through reducing the levels of mitochondrial phosphatidylserine decarboxylase (PISD), which is involved in the synthesis of mitochondrial phosphatidylethanolamine. These observations have uncovered a novel mitochondrial tumor suppressor and demonstrate an interconnection between mitochondrial lipid metabolism and the differentiation program of breast cancer cells, thereby revealing a new mechanism of tumor suppression.

INTRODUCTION

There are currently over 200 different types of cancers, affecting various parts of the body. Cancer can arise in almost any organ and any cell type in the body. While the incidence of certain cancers such as those of the breast, lung, and colon are high, one seldom hears of a diagnosis of heart cancer or skeletal muscle cancer or brain cancer arising from neuronal cells[1]. Surprisingly, these types of cancers are extremely rare or, in some cases, non-existent. This point to the possibility that some tissue-types, and/or specific subset of cells within these tissues have already devised ways of countering neoplasia, and as such, could provide us with novel insights into the prevention and/or treatment of cancer. A characteristic of these "cancer-resistant" cell types (for example, adult myocytes and cardiomyocytes) is that these cells are non-proliferative, terminally differentiated[2,3], and prefer oxidative phosphorylation over glycolysis as their main pathway for energy production. These biological and biochemical characteristics are in stark contrast to those of cancer cells, which are by nature proliferative, relatively undifferentiated, and prefer

glycolysis to oxidative phosphorylation as their primary mode of ATP generation. This led us to hypothesize that the factors that induce or maintain the residence of "cancer-resistant" cells in non-proliferative, differentiated, oxidative-phosphorylation-preferring could have the characteristics of tumor suppressors if expressed in a neoplastic setting. As such, the gene expression profiles of these cells could serve as rich sources of novel tumor suppressors, enabling us to uncover novel dependencies and vulnerabilities of cancer cells. Indeed, this approach of utilizing the gene expression profiles of differentiated muscle cells has allowed us to identify a novel tumor suppressor that operates within mitochondria and negatively affects the growth of a variety of tumor cells *in vivo* and *in vitro* while leaving non-tumorigenic cells minimally affected. Its mechanism of action involves, in significant part, alterations of mitochondrial lipid metabolism that are accompanied by differentiation of cancer cells and their loss of tumorigenicity.

RESULTS

Identification of LACTB as a potential tumor suppressor

C2C12 mouse muscle progenitors and primary human muscle progenitors were differentiated *in vitro* according to standard protocols (see Methods; Extended Data Fig. 1a-c). Gene expression microarray analysis was performed to identify mRNAs that were significantly up-regulated in differentiated post-mitotic muscle cells of both species relative to their undifferentiated, actively cycling counterparts (Extended Data Fig. 1d and Supplementary Table S1). Five genes were chosen for functional validation: adenylate cyclase-associated protein 2 (CAP2), beta-lactamase-like protein (LACTB), receptor accessory protein 1 (REEP1), PDZ and LIM domain protein 3 (PDLIM3), and small muscle protein X-linked (SMPX) (Extended Data Fig. 1e). The cDNA of each was cloned into a doxycycline-inducible Tet-ON vector and expressed in a derivative of the MCF7 human breast cancer cell line that carries an introduced *H-RAS* (G12V) oncogene (MCF7ras)[4]. Upon doxycycline induction LACTB exerted a profoundly negative effect on these cells' ability to proliferate, SMPX exhibited a modest effect, while no significant effect on cell proliferation resulted from over-expression of CAP2, REEP1 or PDLIM3 (Extended Data Fig. 1f and Fig. 2a). Consequently, we focused our attention on characterizing the functional role of the LACTB protein in cancer cells. LACTB is a mitochondrial protein that is related evolutionarily to bacterial penicillin-binding/B-lactamase proteins[5,6]. Homologs of the *LACTB* gene are present in the genomes of all chordates examined thus far. In mammals LACTB appears to be ubiquitously expressed, being most prominent in skeletal muscle, heart and liver[5,7]. Such evolutionary conservation points to an essential, albeit still-unknown, cellular function. LACTB has been found to undergo polymerization into filaments within the mitochondrial inter-membrane space, suggesting that this protein might promote intra-mitochondrial membrane organization[8]. LACTB was also suggested to be involved in the regulation of complex I of the mitochondrial electron transport chain, since down-regulation of LACTB led to decreased activity of this complex[9]. Through gene co-expression analysis and transgenic mice validation, LACTB function has been linked to obesity[10] and fatty acid metabolism[11], hinting at a possible role in regulating cellular metabolic processes.

We performed quantitative RT-PCR analyses in order to compare the levels of *LACTB* mRNA in various non-tumorigenic and tumorigenic mammary cell lines. This analysis failed to show any correlation between LACTB mRNA expression and the neoplastic cell state (Extended Data Fig. 2a). However, since LACTB protein expression has also been shown to be frequently regulated at the post-transcriptional level[12,13,8], we compared the levels of LACTB protein expression in a panel of normal and neoplastic cells. Immunoblot analysis showed a marked reduction of LACTB protein levels in breast cancer cell lines relative to non-tumorigenic mammary cells (Fig. 1a). Out of 18 breast cancer cell lines analyzed 15 showed decreased (albeit never entirely absent) LACTB protein levels, while three cell lines (MCF7ras,

SUM159, MDA-MB-231) cells) exhibited LACTB protein levels comparable to those found in non-tumorigenic cell lines (Fig. 1a). However, further analysis showed that in MCF7ras cells the LACTB protein displayed not only mitochondrial but also some punctate non-mitochondrial staining pattern (Extended Data Fig. 2b) and the LACTB gene from MCF7ras cells contained the R469K mutation in its C-terminus, which is adjacent to one of the three important catalytic/substrate docking domains[6]. The same mutation was found in the endogenous LACTB sequence in SUM159 cells, while the sequence of endogenous LACTB in MDA-MB-231 cells harbored no apparent somatic mutations.

We proceeded to extend our findings with cell lines to clinical samples by examining the expression of LACTB protein in normal human breast tissues (n=120) and in a panel of 714 clinically defined human breast cancer samples. While LACTB was expressed in 100% of normal mammary glands analyzed (Extended Data Fig. 2c), its expression was significantly down-regulated in 34-42% of breast cancer tissues (Fig. 1b, Extended Data Fig. 2d). This down-regulation did not correlate with any particular tumor type, grade or size (Fig. 1b and Extended Data Fig. 2e).

In order to gauge the cell-biological effects of LACTB expression, we induced LACTB in a panel of tumorigenic and non-tumorigenic cell lines and measured cellular proliferation. Since doxycycline has been reported to modulate mitochondrial function via its effects on the mitochondrial translation machinery[14-16], experiments in this study were always performed by adding doxycycline to control cells unless otherwise noted. LACTB over-expression decreased the rate of proliferation in the three breast cancer cell lines tested (HMLER, MCF7ras, HCC1806), while proliferation of the three non-tumorigenic cell lines (HME, MCF10A, and BJ1) was only minimally affected by LACTB over-expression (Fig. 2a and Extended Data Fig. 2f). These results were further confirmed and extended by EdU, Annexin V and Ki-67 staining (Fig. 2b, Extended Data Fig. 2g,h) and provided support for the notion that expression of LACTB caused responding cells to withdraw from the active cell cycle. Of note, the R469K LACTB mutant did not display any negative effect on growth of MCF7ras and HMLER cancer cells (Extended Data Fig. 3a) and when wild-type LACTB was expressed in SUM159 and MDA-MB-231 human breast cancer cells, it had a negative effect on the growth of SUM159 cells but not on the growth of MDA-MB-231 cells (Extended Data Fig. 3b). In summary, these and previous results showed that while a substantial majority of breast cancer cell lines exhibit down-regulated or mutated LACTB and are negatively affected by wild type LACTB induction, there are some breast cancer cell lines (such as MDA-MB-231) that seem to be unaffected by induced LACTB expression.

We then redirected our attention toward determining whether expression of LACTB in already-formed tumors exerted a negative effect on further tumor growth. MCF7ras, HMLER and HCC1806 tumors in which LACTB had been induced decreased significantly in size within 2-3 weeks of LACTB induction, with some tumors completely disappearing by 4 weeks of LACTB induction (Fig. 2c and Extended Data Fig. 3c). Ki-67 staining of tissue sections showed a high proliferation index of the MCF7ras control tumor cells (80% positive), while cells expressing LACTB exhibited lower Ki-67 expression (15% positive) (Extended Data Fig. 3d). This decrease in proliferation was accompanied by an increase in cleaved caspase staining, indicative of apoptosis (Extended Data Fig. 3e). Interestingly, H&E staining of these LACTB-exposed tumors showed a staining pattern that was very different from that of control tumors, particularly for the HMLER and MCF7ras tumors and to a lesser degree for the HCC1806 tumors. Control tumors contained large, poorly differentiated cells with little cytoplasm, relatively little stroma, and small areas of central necrosis. In contrast, tumors expressing induced LACTB consisted of large areas of necrosis extending throughout the entire tumor, abundant stroma, and residual cancer cells were smaller with a more differentiated, epithelial appearance (Extended Data Fig. 3f,d).

In order to extend the evidence that LACTB can play an active role in hindering tumorigenesis, we employed shRNA to down-regulate LACTB expression in HME cells. Two different *LACTB* shRNAs (L-

3 and B-3) were chosen based on their LACTB knockdown efficiencies (86% and 90% respectively; Extended Data Fig. 4a). Decreased levels of LACTB in HME cells (termed HME-shLACTB) resulted in a 2-fold reduction in proliferation relative to unmodified HME cells propagated in parallel, implying that a minimal level of LACTB is necessary for efficient proliferation of non-transformed cells (Extended Data Fig. 4b). The tumorigenic abilities of the two resulting cell populations (denoted HME-shLACTB-L-3 and B-3) were gauged by orthotopic implantation into NOD/SCID hosts. Both cell populations failed to form tumors by 12 weeks post-implantation, indicating that LACTB down-regulation did not suffice, on its own, to transform these cells to a tumorigenic state (Extended Data Fig. 4c). Since loss of tumor suppressor gene function often requires concomitant expression of an oncogene in order for transformation to occur[17-20], we tested whether the concomitant knockdown of LACTB and introduction of an *H-RAS* (G12V) oncogene would make HME cells tumorigenic. Indeed, following orthotopic implantation, these cells formed tumors at 6 weeks post-injection, whereas HME cells expressing only the introduced *H-RAS* (G12V) oncogene failed to form any tumors by 12 weeks post-injection (Extended Data Fig. 4c). We also tested whether decreased levels of LACTB caused tumorigenesis when introduced together with an alternative oncogene. As seen in Extended Data Fig. 4d-h, tumor growth was also observed when down-regulation of LACTB was coupled with up-regulation of an active *c-MYC* (T58A) oncogene but not when coupled with wild-type human HER2 over-expression.

LACTB mechanism of action

Our *in vitro* experiments revealed that carcinoma cells (HMLER, MCF7ras) that experienced and survived forced LACTB expression showed more differentiated morphology characterized by tight epithelial cobblestone islands (Extended Data Fig. 5a), increased expression of markers of epithelial differentiation (Epcam, CD24) and decreased CD44 expression (Fig. 3a). RT-PCR and immunofluorescence analysis confirmed that the cancer stem-cell marker ZEB1[21,22], as well as several mesenchymal markers, were all down-regulated in HMLER cells in which LACTB was induced while epithelial markers were upregulated in these cells (Figure 3b and Extended Data Fig. 5b). Forced overexpression of LACTB in non-tumorigenic HME cells had no discernible effect on their morphology (Extended Data Fig. 5a), the levels of the CD24, CD44 and EPCAM markers (Fig. 3a), and the gene expression profile that monitored the mRNA expression levels of mesenchymal, epithelial and cancer stem-cell markers (Extended Data Fig. 5b). When injected at limiting dilutions into fat pads of NOD/SCID mice, a decrease in tumor formation and tumor weight was seen in the more differentiated HMLER cells that experienced chronic LACTB induction relative to control HMLER cells in which LACTB had not been induced (Extended Data Fig. 5c). Of note, the *in vitro* proliferation rates of these two cell lines were comparable (Extended Data Fig. 5d). To examine whether LACTB expression can directly lead to the induction of epithelial differentiation we used FACS to collect and single-cell clone the more mesenchymal, cancer stem cell (CSC)-enriched $CD44^{high}/CD24^{low}$ cells from a population of HMLER-Tet/ON LACTB cells. LACTB expression was then induced in two of the resulting single-cell clones (HMLER-CD44+ cl.1 and cl.2). In both clonal cell populations, those cells in which LACTB was induced proliferated slower, formed small epithelial islands within 3-4 days of LACTB expression, and exhibited decreased tumorigenesis than the control cells which retained their mesenchymal morphology (Fig. 3c, Extended Data Fig. 5e, Supplemental Videos 1-4). Similar effects on epithelial differentiation coupled with the loss of tumorsphere-forming and tumor-initiating ability were observed in MCF7ras cells forced to express LACTB (Extended Data Fig. 6a-e). Hence, LACTB was capable of inducing epithelial differentiation in carcinoma cells, which was associated with a decrease in their tumor-initiating ability.

To discover more direct mechanism(s) of action of LACTB, we concentrated our attention on examining several mitochondrial processes upon early time points (within 1 day) of LACTB induction.

Such early time points would lead us to the more immediate effects of LACTB on cancer cells, while later responses might represent effects that operate further downstream of LACTB expression. As shown in Extended Data Fig. 7a-f, we failed to detect any significant changes in ATP or ROS production, mitochondrial membrane potential, mitochondrial numbers, localization and structure, or protein levels and localization of markers of mitochondrial fusion and fission in MCF7ras cells in which LACTB expression had been induced for up to 24 hours. However, when these processes were assessed at later time points (3-6 days) they showed significant changes when compared to the control cells (Extended Data Fig. 7a-c,f and data not shown). Since bioinformatics and transgenic mice studies have linked LACTB to obesity and fatty acid metabolism[10,11], we also turned our attention to mitochondrial lipids. LC-MS analysis of mitochondrial lipids showed significant decrease (30-50%) in the quantities of lysophosphatidylethanolamines (LPE) and phosphatidylethanolamines (PE) during the initial induction of LACTB expression (24 hours) in the tumorigenic MCF7ras cells but not in non-tumorigenic HME cells (Fig. 4a, b; Supplemental Table S2). In order to determine whether the lower levels of LPE and/or PE species contributed to LACTB's negative effect on the proliferation of MCF7ras cells, we supplemented the culture medium of LACTB-exposed MCF7ras cell individually with these two lipid classes. Previous publications[23-25] showed that extracellular LPE, but not PE, can be efficiently transported into mitochondria in which the LPE can then be acylated to PE. Supplementation of the growth medium of LACTB-induced MCF7ras cancer cells with 20μM LPE (but not with 50μM phosphatidylethanolamine, phosphatidylglycerol or lysophosphatidylcholine) increased cell proliferation, thus partially reversing the reduction in cell proliferation imposed by LACTB (Fig. 4c). Moreover, EdU staining of MCF7ras cells in which LACTB had been induced in the presence of 20μM LPE exhibited a 50% higher proliferative capacity relative to control MCF7ras (Extended Data Fig. 8a). LC-MS analysis of mitochondrial LPE levels confirmed the increase of LPE levels in mitochondria following supplementation of LPE in culture media (Extended Data Fig. 8b). These data provide support for the notion that a significant part of LACTB-induced negative proliferative effects on MCF7ras cells is mediated by decreased levels of LPE and/or PE. Next, we induced LACTB expression in MCF7ras and HMLER cells that were supplemented with 20μM LPE and monitored the level of CD24 as a marker of cellular differentiation. As seen in Extended Data Fig. 8c, the level of the CD24-differentiation marker in these cancer cells was not increased to that in control cells in which LACTB was induced without LPE addition. These observations indicate that supplementation of these cancer cells with LPE upon LACTB expression can counteract the differentiation-inducing ability of LACTB and that these two events are interconnected.

In order to further identify downstream targets of LACTB's activity, we hypothesized, that LACTB might be modulating the activity of mitochondrial proteins that are involved in phospholipid metabolism, notably LPE and PE biology. One such candidate was mitochondrial phosphatidylserine decarboxylase (PISD), an inner-mitochondrial-membrane enzyme that converts phosphatidylserine (PS) to PE[26]. Figure 5a and Extended Data Fig. 8d show that induced expression of LACTB was associated with strongly decreased levels of the PISD protein (by 60-95%) in mitochondria, while other mitochondrial lipid-metabolizing enzymes were unaffected. This effect was the result of a post-transcriptional event, since PISD mRNA levels were not reduced upon LACTB induction (Extended Data Fig. 8e). Kinetic experiments, in which doxycycline was added to MCF7ras-Tet/ON-LACTB cells for different lengths of time, showed that PISD levels began to decrease at 24 hours of induced LACTB expression (Extended Data Fig. 8f). To determine whether LACTB expression directly reduced PISD function in cells, we supplemented MCF7ras cells with [^{3}H] serine for up to 6 h and monitored the conversion of phosphatidylserine to phosphatidylethanolamine by PISD in both control MCF7ras cells and in MCF7ras cell in which LACTB was induced for 2 days. The cells in which LACTB had been induced showed a significantly reduced (by 60-80%) production of [^{3}H]PE from [^{3}H]PS, consistent with our previous

conclusion that the activity/stability of PISD is influenced by LACTB (Fig. 5b and Extended Data Fig. 8g). Experiments employing the siRNA targeting of PISD showed that down-regulation of PISD exerted similar effects on MCF7ras cell biology as induction of LACTB (Extended Data Fig. 9a-b and as previously described[25]) and that the two effects might operate in the same linear pathway (Extended Data Fig. 9c). Of note, R469K LACTB was unable to downregulate PISD (Extended Data Fig. 9d) and LPE/PE lipid species (Extended Data Fig. 9e and Supplemental Table S2) and more physiological levels of LACTB over-expression (achieved by titrating down our doxycycline levels) in a panel of breast cancer cell continued to negatively affect proliferation of cancer cells, resulted in the down-regulation of PISD levels (Extended Data Fig. 9f,g), caused changes in the levels of mitochondrial LPE/PE lipids and lead to differentiation of HMLER cancer cells (Extended Data Fig. 9h, i).

While LACTB is predicted from its structure to be a serine protease, its proteolytic activity has not been directly confirmed. LACTB is inhibited by Z-AAD-CMK[27], a known granzyme B inhibitor, which led us to predict that it might cleave peptide bonds adjacent to aspartic acid residues. Indeed, LACTB efficiently cleaved the fluorogenic peptide Ac-YVAD-AMC, but not the A-AMC or VP-AMC peptides, indicating that LACTB possesses proteolytic activity and can cleave peptide bonds adjacent to aspartic acid residues (Fig. 5c and Extended Data Fig. 10a). Of note, LACTB R469K mutant also possesses proteolytic activity (Extended Data Fig. 10a). This suggests the possibility that even though this mutant still has proteolytic activity for the ac-YVAD-AMC peptide *in vitro*, its' *in vivo* specificity might have changed due to the posttranslational modification of lysine residue. Our mutational and proliferation studies further determined that the proteolytic function of LACTB and its mitochondrial localization were important for its tumor-suppressing effect and that the PISD down-regulation in the presence of induced LACTB was dependent on the intact catalytic activity of LACTB (Extended Data Fig. 10 b-f). However, *in vitro* incubation of recombinant LACTB with permeabilized mitochondria isolated from MCF7ras failed to result in direct degradation of PISD, suggesting that PISD is not a direct substrate of LACTB (Extended Data Fig. 10g). However, the absence of an effect of LACTB on PISD protein was difficult to interpret, given our inability to know the precise conditions under which these proteins interact *in vivo.* Fig. 5d shows that PISD levels were similarly reduced in response to LACTB induction in tumorigenic HMLER, HCC38, HS578t and to a lesser degree HCC1806 cell lines, but not in the SUM149 breast cancer and the non-tumorigenic HME and MCF10A mammary cell lines. These data indicate that the decrease in the amount of PISD by LACTB might represent a mechanism-of-action of LACTB shared by several, but not all, cancer cells and that this reduction in PISD does not occur in the non-tumorigenic cell lines tested. The inability of LACTB to down-regulate PISD levels in differentiated non-tumorigenic cells (such as HME) explains its inability to negatively affect the proliferation of these cells. This implied, in turn, that in tumorigenic cells that had undergone differentiation and loss of tumorigenicity following LACTB induction the subsequent induction of LACTB might no longer be able to down-regulate PISD. Indeed, as shown in Extended Data Fig. 8f LACTB was unable to down-regulate PISD levels in the already-differentiated MCF7ras cells that were described in Extended Data Fig. 6a-e further supporting our observation that LACTB cannot downregulate PISD in differentiated non-tumorigenic cells, and that the actions of LACTB depend on whether it is induced in the context of less-differentiated cancer cell or fully differentiated non-tumorigenic cells.

DISCUSSION

Our report describes a novel tumor suppressor protein and a novel mechanism of the control of the neoplastic state. We show, using *in vivo*, *in vitro* and breast cancer tissue studies, that LACTB is a potent inhibitor of proliferation of multiple types of breast cancer cells. LACTB has the ability to perturb

mitochondrial lipid metabolism and, through such reprogramming, to modulate the differentiation state of cancer cells. This is achieved via a novel LACTB-PISD-LPE/PE signaling axis (Extended Data Fig.10h).

Mitochondrial phospholipids are important building blocks of mitochondrial membranes and perturbation of PE/LPE might influence diverse processes dependent on the proteins within these membranes. Since LPE can also serve as a precursor of PE within mitochondria, our work does not distinguish between the reduction in PE, LPE, or both as inciting cause(s) for cancer cell differentiation. Some reports have previously linked LPE to cell differentiation. For example, LPE-acyltransferase, the enzyme responsible for synthesis of PE from LPE, has been reported to have a role in cardiac muscle differentiation[28]. LPE also appears to modulate differentiation of neuronal PC12 cells[29]. It is plausible, that changes in the representation of various mitochondrial phospholipids are required for the differentiation of dedifferentiated epithelial cells and that a transient change in the ratio of these mitochondrial phospholipids might initiate the process of differentiation. LPE was also reported to act as an inhibitor of phospholipase D[30]. Since phospholipase D has known roles in inducing cellular differentiation[31,32], down-regulating LPE levels through elevated expression of LACTB, might lead to activation of phospholipase D and subsequent differentiation.

LACTB expression is down-regulated in many breast cancer cell lines and tissues, but is never entirely absent. Indeed, available genomic databases also indicate the absence of any significant numbers of bi-allelic deletions of the *LACTB* gene. Moreover, our shRNA silencing experiments indicate that strong reductions of LACTB leading to the effective absence of this protein negatively affect the growth of normal cells, suggesting that minimal levels of LACTB must be retained in order for normal cell proliferation to proceed. Together, these observations converge on the notion that LACTB may represent an example of tumor suppressor that acts in haploinsufficient way, where loss of the entire protein is deleterious for an incipient cell. Intriguingly, our work shows that LACTB preferentially down-regulates the level of PISD in certain cancer cells while not displaying this ability in the tested non-tumorigenic differentiated cells. Cancer cells differ from normal cells in many biochemical, signaling and regulatory pathways, and it will be interesting to examine which regulatory pathways are responsible for this differential behavior of LACTB, how do the observed changes in mitochondrial PE and/or LPE influence the differentiation state of cancer cells and which signaling pathways are involved in this processes.

Our study opens a novel and still-unexplored cancer research area, in which the study of the expression profiles of "cancer-resistant" tissues may reveal the identities of hitherto-unidentified tumor-suppressing genes. Stated differently, while the vast majority of current cancer research focuses on studying processes leading to tumorigenesis in tissues in which cancer regularly occurs, the research field has overlooked the other side of the coin - to examine mechanisms, including specific genes, operating in tissues where cancers don't occur. Our study shows that we indeed can learn lessons from such cells and tissues and use this knowledge to design new ways of fighting neoplastic cells and the tumors that they form.

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FIGURE LEGENDS

Figure 1. LACTB down-regulation in cancer cells. (a) Western Blot of endogenous LACTB protein levels in a panel of non-tumorigenic and breast cancer cell lines. Note-LACTB is significantly decreased in many breast cancer cell lines, but it is never completely absent. Some levels of LACTB are still present upon over-exposure of Western Blot membrane. NT=non-tumorigenic, BC-L=breast cancer luminal, BC-B=breast cancer basal. (b) Immunohistochemistry of endogenous LACTB protein levels (brown) in a panel of 714 clinically-defined human breast cancer samples. Scores 2 & 3 represent LACTB staining that was considered normal (not downregulated), while scores 0 & 1 represent LACTB staining that was considered downregulated. Lum.A (n=329), lum.B-Her-neg (n=197), lum.B-Her-pos (n=60), Her-pos (n=37), trip-neg (n=91).

Figure 2. LACTB-induced effects on proliferation of breast cancer cells. (a) Proliferation rates of cells upon LACTB induction. Three independent experiments were performed. (b) DNA synthesis in non-tumorigenic and tumorigenic cell lines upon LACTB induction. Numbers within the graphs represent percentages of gated cells. The experiment was repeated twice. (c) Tumor growth rate upon LACTB induction monitored *in vivo* by whole mouse imaging and tumor measurements. P value < 0.0001 (****) is considered very significant. P value>0.05 is non-significant (ns). The experiment was repeated twice.

Figure 3. LACTB-induced effects on cancer cell differentiation. (a) Flow cytometry analysis of marker of stem cells (CD44) and markers of epithelial differentiation (CD24, EPCAM) in tumorigenic HMLER and non-tumorigenic HME cells upon LACTB induction. Numbers within the graphs represent percentages of gated cells. (b) Immunofluorescence staining of HMLER cells upon 2-weeks long LACTB induction. Epithelial markers: E-cadherin (white), β-catenin (white), cytokeratin 8 (CK8, white); mesenchymal markers: vimentin (white), fibronectin (white), stem cell marker Zeb1 (white) and DAPI

(blue). Scale bar 30µm. 2 independent experiments. (c) Growth, morphology and tumorigenicity of two single cell clones upon LACTB induction monitored by live time lapse imaging and orthotopic injections. Videos of clones n.1 and n.2 are shown in supplemental material. Scale bar 200µm. P value < 0.05 is considered significant.

Figure 4. LACTB-induced changes in mitochondrial phospholipids. (a,b) Liquid chromatography-tandem mass spectrometry analysis of mitochondrial lipid content of LACTB-induced (24-hours of induction) vs control HME (a) and MCF7ras (b) cells. The blue perforated line shows the p value of 0.05. Signals above this line have significant $p<0.05$. The experiment was repeated twice. (c) Proliferation rates of MCF7ras and MCF7ras-Tet/ON LACTB cells in the presence of doxycycline with or without supplementation with lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), or lysophosphatidylcholine (LPC) The experiment was repeated three times. Scale bar 200µm.

Figure 5. The role of phosphatidylserine decarboxylase (PISD) in LACTB mechanism. (a) Western blot analysis of sub-fractionated MCF7ras cells and MCF7ras-Tet/ON LACTB-expressing cells with 24 hours of doxycycline treatment. CYT=cytosolic, MITO=mitochondrial fraction. This membrane was also used in Extended Data Fig. 7e. The signal for the control antibodies is shared between these two Figures. (b) Conversion of phosphatidylserine (PS) to phosphatidylethanolamine (PE) in MCF7ras cells and MCF7ras-Tet/ON LACTB treated with doxycycline for 48 h. The experiment was performed in triplicate. P value<0.05 is considered significant. (c) Fluorescent absorbance monitored over time as a measure of active peptidase activity of LACTB. (d) Western blot analysis of PISD levels in mitochondria isolated from a panel of control and Tet/ON LACTB non-tumorigenic (HME and MCF10A) and tumorigenic cell lines where doxycycline was added for 24 h.

METHODS

Cell Culture and reagents

All cells were cultured in a 5% CO2 humidified incubator at 37° C. Cell lines, growth media and sources are described in Table S3. Most of the cell lines were purchased commercially from ATCC or Clonetics. All cell lines used in this study were verified to be mycoplasma negative and their morphology and growth characteristics were compared to published information to ensure their authenticity. For activating tetracycline-inducible gene expression, the cells were treated with 0.05-1µg/ml doxycycline hyclate (Sigma-Aldrich) in media. Fresh doxycycline was added every 3 days.

Muscle Progenitor Cell and Mesenchymal Stem Cell (MSC)

C2C12 mouse myoblast cells (ATCC) were cultured in DMEM+15% FBS. For differentiation of C2C12 cells media were changed to DMEM+2% horse serum. Media was changed every 3 days and differentiation occurred around days 4-6. Human progenitor muscle cells (Cell Applications 151-25a) were grown and differentiated according to manufacturer's protocol.

Animals

All animal experiments were performed with female virgin NOD/SCID mice (non-obese diabetic/severe combined immunodeficiency). Mice were 2-4 months of age at time of injections. A colony of NOD-SCID mice was maintained in-house. Mice were housed and handled in accordance with protocols approved by the Animal Care and Use Committees of the Massachusetts Institute of

Technology. Animals were randomized by age and weight. The exclusion criterion of mice from experiments was sickness of the animals due to the appearance of spontaneous thymomas that sometimes affect NOD/SCID mice. Sample sizes were chosen to reach statistical significance, and tumor measurements and data analysis were performed in a blinded fashion. Maximum tumor size permitted by our IACUC protocol was 2 cm and these limits were not exceeded in any of our experiments.

Viral vectors and infection

Human LACTB cDNA was purchased from Open Biosystems # MHS1010-98051227. REEP1 cDNA was purchased from Open Biosystems # MHS1010-98051276. CAP2, SMPX and PDLIM3 cDNAs were obtained by reverse transcription PCR from differentiated human muscle cells using primers described in Table S4. R469K-LACTB cDNA was obtained by reverse transcription PCR from MCF7ras cells using primers for LACTB described in Table S4. All cDNAs were then subcloned into the FUW-LPT2 tetracycline-inducible lentiviral vector (modified from FUW-tetO by Kong-Jie Kah) with puromycin resistance gene. dS-LACTB was generated by mutating the serine residue in position 164 to isoleucine using primers described in Table S4 and then subcloned into FUW-LPT2 tetracycline-inducible lentiviral vector. d1-97-LACTB was generated by PCR using primers described in Table S4; it is starting with methionine as amino-acid position number 97. c-MYC T58A was purchased from Addgene #18773. RAS-IRES-GFP was purchased from Addgene #18780. Human pWPXL-HER2 plasmid was a kind gift from Wenjun Guo (Albert Einstein College of Medicine, New York). pMMP-LucNeo, carrying coding sequences for luciferase and neomycin phosphotransferase, were obtained from Dr. Segal[33]. To down-regulate LACTB expression we used shLACTB L-3 (Open Biosystems # TRCN0000046625) and shLACTB B-3 (Broad Institute TRC shRNA library # TRCN0000296726). Both shRNAs are cloned in pLKO.1 lentiviral vector with puromycin selection marker. Empty pLKO.1 vector was used as a control. For lentiviral infection, cells were seeded at 30% confluency in 10cm dish and transduced 24 hours later with virus in the presence of 5 µg/ml polybrene (EMD Millipore # TR-1003-G). Cells were then selected by relevant selection marker.

We found the doxycycline-inducible FUW-LPT2 vector to be a little leaky; minimal amounts of cloned gene were expressed even in the absence of doxycycline (as judged by RT-PCR analysis). While this leakiness never had any physiological significance for short term experiments, keeping such cells for a long time in tissue culture might lead to a change in physiology of these cells. Therefore, as a precaution, we never kept cell lines transduced with FUW-LPT2+cloned factor in tissue culture for longer than two months. After two months, the cell lines were discarded and the new ones were freshly transduced with FUW-LPT2+cloned factor vector and selected with proper selection marker.

siRNA and transfection

Four dsiRNA duplexes against human *PISD* gene were designed and purchased from Integrated DNA Technologies.
dsiRNA-PISD-1sense: rCrCrArCrCrArUrCrGrUrGrCrUrCrArUrCrUrUrCrGrArGGC
dsiRNA-PISD-2sense: rCrCrUrArCrCrArArUrGrArCrUrUrCrArGrCrUrUrCrGrUrGAC
dsiRNA-PISD-3sense: rCrArArUrCrArGrGrUrGrGrArGrCrUrGrCrCrArCrArCrUGG
dsiRNA-PISD-4sense: rGrCrArGrGrGrUrGrGrCrUrUrUrGrUrArCrArArGrUrCrAGT
Premade negative control dsiRNA was purchased from Integrated DNA Technologies (DS NC1, 180683394). Cells were plated in 10-cm dishes and next day transfected with Lipofectamine 2000

(ThermoFisher) according to manufacturer's protocol. Analyses were performed 48 hours after transfection.

Tumor cell injections

For orthotopic tumor transplantations, cells were resuspended in 20µl of 50%Matrigel and injected into mammary fat pads of female NOD-SCID mice. The *in vivo* doxycycline treatment was administered through drinking water containing 2 mg/ml doxycycline and 10 mg/ml sucrose.
The tumor incidence and weight were measured 2-3 months post injection. For limiting dilution analyses, the frequency of cancer stem cells in the cell population being transplanted was calculated using the Extreme Limiting Dilution Analysis Program (http://bioinf.wehi.edu.au/software/elda/index.html).
Regarding Fig. 2c: Control MCF7ras cells and MCF7ras-Tet/ON-LACTB cells were injected (10^5 cells per injection) into fat pads of female NOD/SCID mice. When the tumors reached approximately 10mm in diameter doxycycline was added to both groups and tumor growth rate was monitored *in vivo* by whole mouse imaging and tumor measurements.

***In vivo* mouse imaging**

Bioluminescence imaging of luciferase activity was used to monitor tumor growth with a Xenogen IVIS system under 2.5% isoflurane anesthesia at the indicated time points. Imaging of mice was performed by injection of D-luciferin (165mg/kg of body weight, intraperitoneal injection; Caliper Life Sciences) 10 minutes before bioluminescence imaging. Images were analyzed using Living Image Software version 4.3.1 (Caliper Life Sciences).

Immunofluorescence (tissues, cells)

Tumors were fixed in 10% neutral buffered formalin overnight and embedded in paraffin for sectioning. Sections were cut at 5µm. Tumor sections were deparaffinized in Xylene and antigen retrieval was performed in Target Retrieval Solution (Dako # S1700) using a microwave. Background was ten reduced using ImmunoStain Blocker Solution (GeneTex # GTX73323) for one hour at room temperature. Sections were then blocked with 2% normal horse serum in PBS for one hour at room temperature. Sections were incubated with primary antibody at 4^0C overnight. The type, source and dilution of antibodies are described in Table S5. After three washes with PBS, sections were incubated with secondary antibodies (AlexaFluor, Invitrogen, 1:500) for one hour at room temperature, washed three times with PBS, and mounted on glass microslides (VWR#48312-002) in Prolong gold antifade reagent with DAPI (Life Technologies # P36931) and let dry overnight at room temperature in dark.

Cultured cells were seeded on sterilized glass round slides inside 10-cm Petri dishes with cell culture media. Once cells reached a sufficient density, glass slides were transferred into individual wells of 6-well dish and subsequent processing was done in this format at room temperature unless otherwise stated. Cells were washed in PBS, fixed in 10% formalin for 10 minutes, washed once in PBS, permeabilized with PBS+0.1% Triton-X for 10 minutes, washed 3x10 minutes in PBS and incubated in 5% horse serum for 1 hour. Cells were then incubated in specific primary antibodies at 4^0C overnight, washed three times with PBS, then incubated with secondary antibodies for 1 hour at room temperature. After three washes with PBS, stained cells were mounted on glass microslides (VWR#48312-002) in Prolong gold antifade reagent with DAPI (Life Technologies # P36931) and let dry overnight at room temperature in dark. The type, source and dilution of antibodies are described in Table S5.
Immunostained samples were imaged and analyzed using Zeiss RPI Spinning Disc Confocal microscope with MetaMorph Software or Perkin Elmer Spinning Disc Confocal microscope with Volocity software.

Electron microscopy
Cells were fixed in 2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer (pH 7.4), pelleted, and post fixed in 1% OsO_4 in veronal-acetate buffer. The cell pellet was stained on block overnight with 0.5% uranyl acetate in veronal-acetate buffer (pH6.0), then dehydrated and embedded in Embed-812 resin.
Sections were cut on a Leica Ultracut E microtome with a Diatome diamond knife at a thickness setting of 50 nm, stained with uranyl acetate, and lead citrate. The sections were examined using a FEI Tecnai spirit at 80KV and photographed with an AMT ccd camera.

Western Blot
Proteins were extracted from cells using RIPA buffer (Sigma Aldrich # R0278) always in the presence of Protease (Roche Diagnostics # 11836153001) and Phosphatase (Roche Diagnostics # 04906845001) Inhibitors. Human Skeletal Myoblast lysate was purchased from ZenBio # TCE-SKB and lysate from differentiated human skeletal myotubes was purchased from ZenBio # TCE-SKM. Approximately 10-20µg of protein lysate was loaded on the gels. Western blots and transfers were done using standard protocols. Horseradish peroxidase-conjugated secondary antibodies were used (Cell Signaling, 1:5000). Blots were developed using ECL (Dura or Femto, Thermo Scientific). The type, source and dilution of antibodies are described in Table S5.

Western Blot analysis related to [^{3}H]serine labeling of phospholipids was performed by loading sample proteins on a 10% gel, then transferring the proteins to polyvinylidene difluoride membranes and blocking in 1% milk. The antibodies and their dilutions are described in Table S5. HRP substrate is Luminata Forte (Millipore) and images were collected on G:BOX with Syngene software.

Quantitative RT-PCR
Total RNA was isolated directly from cultured cells using the RNeasy Plus Mini kit (Qiagen # 74136). Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Life Technologies # 4368814). mRNA levels were measured with gene-specific primers using the SYBR Green I master mix (Roche) on a Roche LightCycler 480 system (Roche). Relative expression levels were normalized to GAPDH. The PCR primer sequences are listed in Table S4.

Clinical data in human breast tissues
LACTB expression was studied on 808 patient samples assembled on a tissue microarray as previously described[34]. The study was approved by the ethical committee of the Kanton Zürich under reference number KEK-ZH-Nr. 2014-0604. Tumor tissue was immunohistochemically stained using the LACTB polyclonal antibody (Proteintech Group, dilution 1:200) on an automated immunostaining platform (Leica BOND, Wetzlar, Germany). Immunohistochemical intrinsic subtyping of the cohort was performed as previously described[35]. LACTB expression was analyzed as no expression or lower than in normal tissues (scores 0, 1) and normal expression in the luminal or basal compartment (scores 2, 3). Statistical analysis was performed with SPSS statistical package version 21 (IBM P-values < 0.05 were considered statistically significant).

Flow Cytometry (Edu, Annexin, ROS and Mitochondrial Membrane Potential)
Cells were trypsinized and filtered through 40µm cell strainers to obtain single cells. Cells were then labeled for flow cytometry by 1 hour incubation with dye-conjugated antibodies and washed once in PBS.

The list of antibodies, their source and dilution is in Table S5. Samples were sorted on a BD FACSAriaII sorter and analyzed using BD FACSDiva Software (BD Biosciences).

Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technologies # C10424) was used for Edu staining according to manufacturer's protocol.
ANXN V-PE Apoptosis Detection Kit (BD Biosciences # 559763) was used for annexinV staining according to manufacturer's protocol.
MitoProbe DiIC 1 (5) Assay Kit (Life Technologies # M34151) was used for mitochondrial membrane potential staining according to manufacturer's protocol.
DCFDA - Cellular Reactive Oxygen Species Detection Assay Kit (Abcam # ab113851) was used for ROS measurement according to manufacturer's protocol.

ATP measurement
ATP levels within cells were assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega # G7572) according to manufacturer's protocol. 1000-3000 cells were seeded in a flat bottom 96-well plate. Four replicates were plated for each time point.

Soft agar colony formation assay (tumorspheres)
Growth in soft agar was performed as described in[36].

Subfractionation and Mitochondrial Isolation
Cells were harvested and mitochondria isolated using the Qproteome Mitochondria Isolation Kit (Qiagen # 37612) for mitochondrial lipid composition analysis or Cell Fractionation Kit (Abcam # ab109719) for Western Blot subfractionation experiments. Isolated mitochondria were frozen at -80°C. The Cell Fractionation Kit was also used for isolating cytosolic fractions.
Permeabilized Mitochodria: Mitochondria isolated using Qproteome Mitochondria Isolation Kit were incubated in hypotonic 100 mM HEPES (pH 7.0-7.6) solution (Sigma # H0887) with or without 150 mM NaCl.

Mitochondrial Lipid Extraction
Frozen mitochondria in eppendorf tubes were thawed on ice, after which 600µl LC/MS grade 100% methanol, 300µl LC/MS grade water, 400µl chloroform (without amylenes) were added in this order. Samples were vortexed for 10 min at 4°C and centrifuged at 13000 RPM for 10 min at 4°C. The lower lipid-containing layer was then carefully collected and dried for 1 h. Lipid extracts were stored at -80°C.

Liquid Chromatography/Mass Spectrometry
Lipid extracts were separated on an Ascentis Express C18 2.1 x 150 mm 2.7µm column (Sigma-Aldrich, St. Louis, MO) connected to a Dionex UltiMate 3000 UPLC system and a QExactive benchtop orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a heated electrospray ionization (HESI) probe. Dried lipid samples were typically dissolved in 50µl 65:30:5 acetonitrile:isopropanol:water (v/v/v) and 5µl was injected into the LC/MS, with separate injections for positive and negative ionization modes. Mobile phase A in the chromatographic method consisted of 60:40 water/acetonitrile in 10mM ammonium formate and 0.1% formic acid, and mobile phase B consisted of 90:10 isopropanol/acetonitrile, also with 10 mM ammonium formate and 0.1% formic acid. The chromatographic gradient was described previously[37]. The column oven and autosampler tray were held at 55°C and 4°C, respectively. The MS instrument parameters were as described previously[38] and

modified by[39]. The spray voltage was set to 4.2 kV, and the heated capillary and the HESI were held at 320°C and 300°C, respectively. The S-lens RF level was set to 50, and the sheath and auxiliary gas were set to 35 and 3 units, respectively. These conditions were held constant for both positive and negative ionization mode acquisitions. External mass calibration was performed using the standard calibration mixture every 7 days. MS spectra of lipids were acquired in full-scan / data-dependent MS^2 mode. For the full scan acquisition, the resolution was set to 70,000, the AGC target was 1e6, the maximum integration time was 50 msec, and the scan range was m/z = 133.4-2000. For data-dependent MS^2, the top 10 ions in each full scan were isolated with a 1.0 Da window, fragmented at stepped normalized collision energy of 15, 25, and 35 units, and analyzed at a resolution of 17,500 with an AGC target of 2e5 and a maximum integration time of 100 msec. The underfill ratio was set to 0. The selection of the top 10 ions was subject to isotopic exclusion, a dynamic exclusion window of 5.0 sec, and an exclusion list of background ions based on a solvent blank.

High-throughput identification and relative quantification of lipids was performed separately for positive and negative ionization mode data, using LipidSearch software (Thermo Fisher Scientific / Mitsui Knowledge Industries) with the default parameters for QExactive Product Search and Alignment. After alignment, raw peak areas for all identified lipids were exported to Excel and filtered according to the following pre-determined quality control criteria: Rej ("Reject" parameter calculated by LipidSearch software) equal to 0; PQ ("Peak Quality" parameter calculated by LipidSearch software) greater than 0.85; CV (standard deviation / average peak area across triplicate injections of a representative [pooled] biological sample) below 0.4; R (linear correlation across a three-point dilution series of the representative [pooled] biological sample) greater than 0.9. Typically ~70% of identified lipids passed all four quality control criteria. Raw peak areas of the filtered lipids were added to generate a "total lipid signal" for each sample, and individual lipid peak areas were normalized to this total signal as a control for extraction efficiency and sample loading. These normalized lipid abundances from control and LACTB-expressing cells were used to calculate fold changes and p-values (Student's t-test, two-tailed, unequal variance) upon LACTB expression. After this calculation, positive and negative ionization mode data were combined to generate Fig. 4a and 4b.

Lipid Supplementation

All lipids were purchased from Avanti Polar Lipids: lysophosphatidylethanolamine (LPE, VWR # 100123-538), lysophosphatidylcholine (LPC, Avanti Polar Lipids # 830071P), phosphatidylethanolamine (PE, VWR # 100127-544), phosphatidylglycerol (PG, VWR # 100123-722). A stock solution (25mM) of LPE was prepared as described in[40]. Stock solutions of LPC, PE and PG (25-100mM) were made by dissolving these lipids in chloroform:methanol:water (65:35:8) (v:v) as advised by Avanti Lipids. The lipids were replenished every 1 to 3 days.

Time lapse imaging

Cells were plated sparsely in 12-well plates. If doxycycline was added, it was added 1 h before imaging. Cells were monitored with 10x objective using Nikon TE2000 automated inverted microscope with incubation enclosure (37^0C, 5%CO2). MetaMorph acquision software was used to analyze the data.

Recombinant LACTB preparation

The open-reading frame encoding LACTB was transferred into a Gateway-compatible version of the pCLNCX vector (Imgenex) containing C-terminal FLAG and His tags and HEK293T cells were stably infected according to the manufacturer's instructions. The R469K mutant was generated using the

QuikChange Site Directed Mutagenesis kit (Stratagene). Cells were grown in DMEM with 10% fetal bovine serum at 37°C with 5% CO_2. Infected cells were selected with media containing hygromycin (100µg/mL) and grown to 100% confluency (10 × 15 cm plates total). For isolation of LACTB, cells were washed two times with PBS and scraped. Cell pellets were then isolated by centrifugation at 1,400 × *g* for 3 min. The pellets were resuspended in RIPA buffer (5mL, Cell Signaling Technology), sonicated, and debris was removed by centrifugation at 12,000 × *g* for 15 min. LACTB was then captured by incubation overnight with 300µL of Anti-FLAG M2 affinity gel (Sigma). The agarose gel was washed three times with PBS (5mL), and LACTB was eluted with 1mL of 150ng/µL solution of 3X FLAG peptide (Sigma) in PBS. Protein was then concentrated (and FLAG peptide removed) using an ultra-centrifugal filter unit (Amicon, 30kDa cut-off) according to manufacturer's instructions. Protein concentration was determined using a protein assay kit (Bio-Rad). Glycerol was added to a final concentration of 10%, and proteins were aliquoted stored at -80 °C until use.

LACTB *in vitro* substrate assay

LACTB (15nM final concentration) was added to 96-well black, clear-bottom plate (Costar # 3603) in 99µL PBS. Substrates were dissolved in DMSO at 100x indicated concentration (see below) and 1µL was added to each well. Reactions were incubated for 1 h at 25°C while been read on a Spectramax M5 plate reader (Molecular Devices) using an excitation wavelength of 380 nm and an emission wavelength of 460nm. To test for cleavage of the substrates Ac-YVAD-AMC, A-AMC, and VP-AMC, a final concentration of 100µM of each substrate was used. To assess kinetics of Ac-YVAD-AMC cleavage, final substrate concentrations of 100, 33, 11, 3.7, 1.23, 0.41, and 0.14µM were used. The initial reaction velocities for each concentration were fit to a nonlinear regression (Prism software).
Related to Extended Data Fig. 10g: Recombinant LACTB (0.2 µg) was mixed with perforated mitochondria (1 µg) in PBS and incubated at 25°C for 2 hours.

[^{3}H]serine labeling of phospholipids in cells

MCF7ras and MCF7ras-Tet/ON-LACTB cells were plated on 6-cm dishes and treated with doxycycline for 48 hours. Pulse medium (20ml DMEM + 10µl dox + 100µl [^{3}H]serine) was then added for 2, 4 and 6 h. The [^{3}H-serine] was purchased from Perkin Elmer (250uCi/ 250 µl). Cells were harvested, lysed and lipids were extracted and dried. The resuspended lipid extract (50/75µl) was applied to thin-layer chromatography plates (Millipore) and separated in the solvent system chloroform:methanol:acetic acid:formic acid:water (70:30:12:4:1, v:v). Bands corresponding to standard PS and PE were scraped from the plates and radioactivity was measured by scintillation counting after overnight incubation at room temperature. The experiment was performed in triplicate.
Regarding Fig. 5b: Of note, the amount of [^{3}H]PS was not altered by LACTB expression. This is in agreement with numerous reports showing that the overall phospholipid levels of cells, including those of PS, are very tightly regulated[41,42]. As such, when PS levels increase, the rate of endogenous PS biosynthesis is quickly reduced in a compensatory manner.

Microarray analysis

The microarrays used were Human and Mouse Agilent 4x44k arrays. Arrays were scanned using an Agilent scanner and the data was extracted using Agilent's Feature Extraction software. Agilent two-color arrays were normalized within-array by loess and between-arrays by quantile normalization of average intensities (Aquantile), as implemented by the limma package in Bioconductor. Replicate spots were averaged and then mean-centered to obtain log ratios. Human and mouse orthologs were obtained from Ensembl BioMart and Homologene.

Proliferation assay
Proliferation of cells was assessed using the WST-1 Cell Proliferation Reagent (Roche Diagnostics # 11644807001) according to manufacturer's protocol. Cells (1000-3000) were seeded in a flat bottom 96-well plate. Four replicates were plated for each time point.
Proliferation of cells was also assessed by seeding cells in 10-cm Petri dishes and trypsinization and manual counting of cells, using either haemocytometer or Vi-CELL XR Cell Viability analyzer (Beckman Coulter), at indicated time points.

Statistical analysis and Abbreviations
All the data are presented as the mean ± standard errors of mean (SEM) unless otherwise specified. An unpaired two tailed Student's t-test was used to calculate the p values except as otherwise specified. $p<0.05$ was considered significant. $0.05>p>0.01$ (*); $0.01>p>0.001$ (**); $0.001>p>0.0001$ (***); $p<0.0001$ (****). Abbreviations are described in Table S6.

Data Availability
The microarray dataset was submitted to GEO databank (GSE72916). Source data for Western Blots, tumor data and LC-MS experiments are included in Supplementary Information. More detailed data and/or protocols are available upon request from the corresponding author.

EXTENDED REFERENCES (related to Methods)

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AUTHOR CONTRIBUTIONS

Z.K. conceived the project, designed and performed the experiments, analyzed data and prepared the manuscript. J.L.D. provided technical support to Z.K. J.D.C. performed the kinetic and proliferation experiments and data analysis. E.F. performed LC/MS experiments and data analysis. S.L. and J.E.V. designed and performed radioactive labeling experiments and analyzed data. D.A.B., M.C.O. and T.R.G designed, performed and analyzed the LACTB cleavage experiments. B.B. provided technical support. V.T and A.N. designed, performed and analyzed histopathology studies. F.R. performed mice surgeries and tumor growth monitoring and quantifications. P.T analyzed microarray studies. R.A.W. designed and supervised this study and prepared the manuscript.

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Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Z.K. (keckesov@wi.mit.edu) or R.A.W. (weinberg@wi.mit.edu).

EXTENDED DATA FIGURE LEGENDS

Extended Data Figure 1. Identification of potential tumor suppressors. (a) Light microscope images of undifferentiated and differentiated human muscle progenitor cells and mouse C2C12 muscle progenitor cells. Scale bar 200μm. (b) Immunofluorescence analysis of mouse C2C12 cells undergoing differentiation. Cells were stained with marker of skeletal muscle differentiation α–actinin (green), actin-staining agent phalloidin (red) and DAPI (blue). Scale bar 30μm. (c) qRT-PCR analysis of expression levels of several known tumor suppressors and cell-cycle inhibitors in differentiated human skeletal muscle cells confirming that these cells abundantly expressed a variety of tumor suppressors. All the

values are relative to undifferentiated cells. GAPDH expression was used as a normalization control. Experiment was performed in duplicate. (d) Microarray analysis of undifferentiated (UD) and differentiated (D) skeletal muscle cells of human and mouse origin (87 genes, $p<0.01$, fold change>2). (e) qRT-PCR analysis of mRNA levels of five candidate genes. Values are relative to undifferentiated human skeletal muscle progenitor cells. GAPDH expression was used as a normalization control. Experiment was performed in duplicate. (f) Light microscope images of MCF7ras cells transduced with five doxycycline-inducible factors in the absence or presence of doxycycline (DOX). Images were taken after 12 days of DOX treatment in all groups except for the LACTB cells which were treated for 6 days with DOX. Scale bar 200µm.

Extended Data Figure 2. LACTB expression in normal and neoplastic cells. (a) qRT-PCR analysis of endogenous LACTB mRNA levels in non-tumorigenic (HME, MCF10A) and neoplastic breast cell lines. All values are relative to those in the non-tumorigenic HME cells. GAPDH expression was used as a normalization control. Experiment was performed in duplicate. (b) Immunofluorescence staining of LACTB in non-tumorigenic (MCF10A) and tumorigenic (MCF7ras) cell lines. Cells were stained with mitochondrial marker (green), LACTB (red) and DAPI (blue). The experiment was performed in triplicate. Scale bar 30µm. (c) Immunohistochemistry of LACTB protein levels (brown) in normal human mammary glands. (d) Immunohistochemistry of endogenous LACTB expression levels (brown) in human breast cancer tissue sections. Shown is the amount of LACTB in normal mammary gland and in the adjacent neoplastic mammary gland. DCIS=ductal carcinoma in situ; Invasive=invasive carcinoma (red circles), M=macrophages (yellow circles); BV=blood vessel. (e) Stratification of low and high levels of LACTB in human breast cancer clinical samples of different grade, size and nodal stage. (f) Immunoblotting of exogenous LACTB protein in control cells (C) and cells in which LACTB was induced by doxycycline (DOX) for 2 days (L). (g) AnnexinV staining in non-tumorigenic (HME) and tumorigenic (HMLER, MCF7ras, HCC1806) cell lines upon LACTB induction. Numbers within the graphs represent percentages of gated cells. (h) Immunofluorescence analysis of control MCF7ras cells mixed with MCF7ras-Tet/ON LACTB cells, in which LACTB was induced for 3 days. Proliferation marker KI-67 (green), LACTB (red), and DAPI (blue). Note the mutually exclusive KI-67 and LACTB staining in these cells. Scale bar 30µm.

Extended Data Figure 3. LACTB-induced effects on proliferation of breast cancer cells. (a) Proliferation curves of MCF7ras and HMLER cells over-expressing wild type (wt) and R469K LACTB. (b) Proliferation curves of SUM159 and MDA-MB-231 cells upon LACTB induction. (c) Tet/ON LACTB cells were injected (10^5 cells per injection for HCC1806 cells and 10^6 cells per injection for HMLER cells) into fat pads of female NOD/SCID mice. HCC1806-control tumors (n=11), HCC1806+LACTB tumors (n=15). When the tumors reached approximately 5mm in diameter, mice were randomly divided into two groups and doxycycline was added to one group. *In vivo* whole mouse images are shown for HCC1806 tumors. Tumor weight and number of resulting tumors was measured at 3 weeks of doxycycline treatment. P values < 0.01 are considered very significant (**). (d) Immunofluorescence analysis of tissue sections of control and Tet/ON LACTB MCF7ras tumors with 1 week (Tet/ON LACTB) or two weeks (control and Tet/ON LACTB) of doxycycline induction. Tissues were stained with the cell proliferation marker KI-67 (green), LACTB (red), and DAPI (blue). Note the mutually exclusive effects of LACTB induction on KI-67 staining in the middle panel. Scale bar 30µm. (e) Immunofluorescence analysis of tissue sections of MCF7ras, and MCF7ras-Tet/ON LACTB tumors in which doxycycline (DOX) was added to both groups for 1 or 2 weeks. Tissues were stained with antibodies against a marker of apoptosis (cleaved caspase, white), and DAPI (blue). Staining was quantified in 8-15 images for each group. “ns” not significant,

"*"significant (0.05> p >0.01), "**"very significant (p < 0.01). Scale bar 30µm. (f) H&E staining of MCF7ras, HCC1806, and HMLER tumors without or with 2- or 3- weeks of *in vivo* LACTB induction. Scale bar 200µm.

Extended Data Figure 4. Collaboration between down-regulated LACTB and oncogene expression in cellular transformation. (a) Immunoblotting of endogenous LACTB protein in HME cells transduced with different short hairpin (sh) RNA vectors directed against LACTB. Non-tumorigenic HME cells are included as a positive control and tumorigenic HMLER cells as a negative control for LACTB expression. Highlighted in red are two LACTB shRNAs chosen for further study. (b) Proliferation rates of HME cells transduced with different LACTB-shRNAs. (c,d,e) Tumor incidence was monitored, by *in vivo* imaging, in non-tumorigenic HME cells and in HME cells transduced with shLACTB vectors (L-3 or B-3) with or without concominant expression of *H-RAS* (G12V)(c), *c-MYC* (T58A)(d) or wild type human *Her2*(e) oncogene. Mice were monitored 6, 9 and 12 weeks post-injection. IN=small indolent tumors which spontaneously regressed. (f,g) Western Blot analyses of H-Ras, c-myc and wt Her2 expression levels in HME-derived cell lines compared to control HME cells.

Extended Data Figure 5. LACTB-induced effects on HMLER differentiation. (a) Light microscope images of HMLER and HME cells upon LACTB induction. Scale bar 200µm. (b) qRT-PCR analysis of relative mRNA levels of mesenchymal, stem cell and epithelial markers in tumorigenic HMLER and non-tumorigenic HME cells upon LACTB induction. All the values are relative to control HMLER or HME cells in which LACTB was not induced. GAPDH expression was used as a normalization control. (c) Frequency of cancer stem cells in control HMLER cells and in differentiated HMLER cells where LACTB was induced *in vitro* for two weeks. Cells were injected in limiting dilutions ($1x10^6$, $5x10^5$, $1x10^5$) into fat pads of female NOD/SCID mice. 8 weeks post-injection mice were euthanized and tumor frequency and tumor diameter calculated and measured. Diameters of tumors arising in $1x10^6$ group are shown. $p<0.001$ is considered very significant (***). (d) Proliferation curves of control HMLER cells and differentiated HMLER cells. (e) Time lapse images of HMLER-Tet/ON LACTB $CD44^{high}CD24^{low}$ single cell clone 2 with (+DOX) or without (NO DOX) LACTB induction. Scale bar 200µm. Videos of clones n.1 and n.2 are shown in supplemental material.

Extended Data Figure 6. LACTB-induced effects on MCF7ras differentiation. (a) Light microscope images of control MCF7ras cells and two independently derived MCF7ras bulk populations that survived 2-weeks long LACTB induction and re-entered their proliferation cycle (LACTB survivor 1 and 2). LACTB survivor cells displayed more epithelial-like, differentiated morphology, characterized by tight cobblestone epithelial features. Scale bar 200µm. (b) Flow cytometry analysis of levels of the epithelial differentiation marker (CD24) in control MCF7ras cells, MCF7ras cells in which LACTB was induced for 3 days and two independently derived MCF7ras bulk populations that survived 2-weeks long LACTB induction and re-entered their proliferation cycle (LACTB survivor 1 and 2). (c) Proliferation curves of control MCF7ras cells and two independently derived MCF7ras bulk populations that survived 2-weeks long LACTB induction and re-entered their proliferation cycle (LACTB survivor 1 and 2). (d) Quantification of *in vitro* tumor sphere formation of control MCF7ras cells and two independently-derived MCF7ras-LACTB survivor populations. Experiment was repeated twice. $P<0.01$ (**); $p<0.001$ (***). Scale bar 200µm. (e) *In vivo* tumorigenicity and cancer stem cell frequency of control MCF7ras cells and two independently-derived MCF7ras-LACTB survivor populations. Cells were injected at limiting dilutions ($1x10^3$, $1x10^2$) into fat pads of female NOD/SCID mice and tumor formation was monitored by *in vivo* imaging 8-weeks post-injection.

Extended Data Figure 7. LACTB-induced effects on mitochondrial functions. (a) Measurements of ATP levels in MCF7ras cells upon LACTB induction. (b) Measurements of ROS levels in MCF7ras cells upon LACTB induction. Numbers within the graphs represent percentages of gated cells. (c) Measurements of mitochondrial membrane potential, through incorporation of cyanine dye $DiIC_1(5)$, by flow cytometry in MCF7ras cells upon LACTB induction. Numbers within the graphs represent percentages of gated cells. (d) Immunofluorescence analysis of control MCF7ras cells mixed with MCF7ras-Tet/ON LACTB cells, where LACTB was induced by addition of doxycycline for 1 day. Cells were stained with mitochondrial marker (green), LACTB (red), and DAPI (blue). Mitochondrial signal per area in control cells (n=16) and in LACTB-expressing cells (n=17) was calculated by ImageJ software. p value > 0.05 is considered not significant (ns). Scale bar 30μm. (e) Western blot analysis of sub-fractionated control MCF7ras cells and MCF7ras-Tet/ON LACTB-expressing cells with 24 hours of doxycycline treatment. CYT=cytosolic, MITO=mitochondrial fraction. Membrane was probed for proteins involved in mitochondrial fusion (Opa1, Mfn1, Mfn2), fission (Fis1, Drp1), composition of respiratory chain (individual OXPHOS components) and control antibodies: LACTB (to show the proper induction and localization of LACTB), actin (cytosolic marker) and COX4 (mitochondrial marker). The membrane in this Extended Data Figure was also used in Fig. 5a, where it was probed with different set of antibodies. Therefore the signal for the control antibodies is shared between these two Figures. (f) Electron microscopy images of mitochondria in control MCF7ras cells or MCF7ras cells where LACTB was induced for 1 or 3 days. Scale bar 600nm.

Extended Data Figure 8. The role of PISD in LACTB mechanism-I. (a) Measurement of DNA synthesis (through EdU incorporation) in MCF7ras-Tet/ON LACTB cells upon LACTB induction with or without supplementation of growth media with 20μM LPE. (b) LC-MS analysis of mitochondrial LPE levels upon supplementation of MCF7ras cells with 20μM LPE for 24 hours. (c) Expression levels of CD24 differentiation marker in MCF7ras and HMLER cells upon LACTB induction for 6 or 9 days respectively, with or without supplementation of growth media with 20μM LPE. (d) Raw western blot image showing PISD subcellular location and levels in sub-fractionated MCF7ras and MCF7ras-Tet/ON LACTB cells (related to Fig. 6a). Doxycycline was added to both cell lines for 24 h. (e) qRT-PCR analysis of mRNA levels of LACTB and PISD in control MCF7ras cells and MCF7ras cells in which LACTB was induced for 3 days. GAPDH expression was used as a normalization control. (f) Time course analysis of levels of LACTB and PISD in control MCF7ras and MCF7ras-Tet/ON LACTB cells in which LACTB was induced by addition of doxycycline (DOX) for the indicated times. In control MCF7ras cells doxycycline was added for 3 days. Also shown are the PISD and LACTB levels in MCF7ras-Tet/ON-LACTB differentiated survivor cell populations 1 and 2 where DOX was added for 24 hours. (g) Related to Fig. 5b. MCF7ras cells (C) and MCF7ras-Tet/ON LACTB cells (L) were incubated in the presence of doxycycline for 48 h. [^{3}H]serine-containing media was then added for 2, 4 or 6 h. A portion of cell lysate was analyzed by immunoblotting to confirm expression of LACTB, down-regulation of PISD and equal protein levels in the samples (by calnexin). Western Blot analysis of control MCF7ras cells and MCF7ras cells transfected for 48 h with four different PISD-siRNAs.

Extended Data Figure 9. The role of PISD in LACTB mechanism-II. (a) Western Blot analysis of control MCF7ras cells and MCF7ras cells transfected for 48 h with four different PISD-siRNAs. (b) The proliferation ability of MCF7ras cells transfected with different PISD siRNAs was measured by EdU staining using FACS. (c) Control MCF7ras and MCF7ras-Tet/ON-LACTB cells were treated with doxycycline for 2 days with or without concominant transfection with 4 different PISD siRNAs. The

proliferation ability of the cells was measured by EdU staining using FACS. The rectangle represents the gate containing proliferative cells. (d) LACTB and PISD protein levels in mitochondrial fractions of control MCF7ras cells and MCF7ras cells with 1 day of wild type LACTB or LACTB R469K induction. (e) LC-MS analysis of mitochondrial PE/LPE species (that were shown to be down-regulated upon wild type LACTB induction) in control MCF7ras cells and MCF7ras cells where LACTB R469K mutant was induced for 24 hours. Values are shown in Supplementary Table S2. (f) Proliferation curves of HMLER, and HCC1806 cells upon addition of 0.05μg/ml DOX. (g) LACTB and PISD levels in non-tumorigenic HME and tumorigenic HMLER and HCC1806 cells upon addition of 0.05μg/ml DOX. (h) LC-MS analysis of mitochondrial PE/LPE/cardiolipin species in control HMLER cells and HMLER cells where lower levels of LACTB were induced, through addition of 0.05μg/ml DOX for 24 hours. Values are shown in Supplementary Table S2. (i) FACS analysis of CD44 levels in HMLER and HMLER-Tet/ON-LACTB upon addition of 0.05μg/ml DOX for 14 days.

Extended Data Figure 10. LACTB mutagenesis. (a) Related to Fig. 5c. Velocity of the ac-YVAD-AMC enzymatic reaction in relation to substrate concentration for wild type- and R469K mutant LACTB. (b) Comparison of amino-acid sequence of wild type (wt) LACTB, dS-LACTB (catalytic site LACTB mutant where critical serine residue was replaced by an isoleucine) and d1-97-LACTB (mitochondrial localization mutant, as described in ref. 8). Only partial sequence of LACTB is shown. The points of mutation of dS- and d1-97 LACTB are highlighted in red and marked by red star symbol. Blue star symbol marks the site of R469K mutation in endogenous LACTB from MCF7ras and SUM159 cells. Green star symbol marks the site of important substrate docking site in LACTB. (c) Immunofluorescence analysis of MCF7ras-Tet/ON dS-LACTB and MCF7ras-Tet/ON d1-97-LACTB cells, where doxycycline was added for 24 hours. Cells were stained with mitochondrial marker (green), LACTB (red), and DAPI (blue). Scale bar 30μm. (d) Western blot analysis of expression levels of LACTB in control MCF7ras cells and MCF7ras-Tet/ON LACTB (wild type, dS, d1-97, dSISK) cells where doxycycline was added for 24 hours. dSISK-LACTB mutant harbors deletion of 4 amino acid residues in catalytic site of LACTB. The expression level of this mutant was unstable therefore we did not include this mutant in our study. (e) Proliferation rates of control MCF7ras cells and MCF7ras-Tet/ON LACTB (wt, dS, d1-97) cells upon addition of doxycycline for indicated number of days. Pictures were taken at 6 days of doxycycline induction. Scale bar 200μm. (f) Western blot analysis of PISD expression in mitochondria isolated from MCF7ras and MCF7ras-Tet/ON LACTB (wt and dS) cells where doxycycline was added for 24 hours to all groups. (g) Western blot analysis of PISD levels after *in vitro* incubation of permeabilized mitochondria (isolated from MCF7ras cells) with or without addition of recombinant LACTB (isolated from HEK293 cells). (h) Graphical Abstract**.** LACTB induction leads to a change in cancer cell state. As such, a proliferative, less differentiated cancer cell turns into a non-tumorigenic differentiated cancer cell upon LACTB induction. This is manifested by an initial disappearance of Ki-67 proliferation marker, followed by down-regulation of CD44 stem-cell marker and increased expression of CD24 and EPCAM differentiated epithelial marker. This is achieved via the ability of LACTB to decrease the protein expression levels of mitochondrial PISD enzyme and the subsequent changes in mitochondrial phosphatidylethanolamine and/or lyso-phosphatidylethanolamine levels.